METHODS FOR IDENTIFYING CANDIDATE COMPOUNDS FOR TREATING, REDUCING, OR PREVENTING PATHOGENIC INFECTIONS

Background of the Invention

The invention relates to drug-screening assays for evaluating and identifying compounds capable of affecting pathogenicity and virulence of a pathogen.

Pathogens employ a number of genetic strategies to cause infection and, occasionally, disease in their hosts. The expression of microbial pathogenicity is dependent upon complex genetic regulatory circuits. Knowledge of the themes in microbial pathogenicity is necessary for understanding pathogen virulence mechanisms and for the development of new "anti-virulence" or "anti-pathogenic" agents, which are needed to combat infection and disease.

The mechanism of pathogenesis and the host defense is a field of intense investigation. Antibiotics have been an effective tool to treat unwanted bacterial infections. However, due to the increasing incidence of resistance to current antibiotics, new antibiotics are needed. Antibiotics that target non-essential genes are desirable because there is limited, if any, selection pressure on these genes since they are not required for the survival of the bacteria. Thus, bacteria are less likely to develop resistance to antibiotics that target these genes.

In nature, most bacteria live not as individual cells but as pseudo-multicellular organisms that coordinate their population behavior via small extracellular signal molecules. Under appropriate conditions, these molecules are released into the environment, taken up, and responded to by surrounding cells (Fuqua et al., *Annu. Rev. Genet.* **35,** 439-68, 2001; Miller et al., *Annu. Rev. Microbiol.* **55,** 165-199, 2001; Withers et al., *Curr. Opin. Microbiol.* **4,** 186-193, 2001). 'Quorum sensing' (QS), is the archetypal intercellular communication system used by many bacterial species to regulate their gene expression in response to cell density. Using this regulatory system, all the individual bacterial cells behave coordinately and synergistically as a community, for example, in growth dynamics and resource utilization (Fuqua et al., *J.*

Bacteriol. **176,** 269-275, 1994). A common feature of all QS systems is the transcriptional activation and repression of a large regulon of QS-controlled genes when a minimal threshold concentration of a specific autoinducer is reached.

The QS system used by Gram-negative bacteria is mediated by the extracellular signaling molecules, *N*-acyl-L-homoserine lactones (AHLs) (Withers et al., *Curr Opin Microbiol* **4,** 186-193, 2001; Fuqua et al., *Annu Rev Genet* **35:** 439-68, 2001). The versatile and ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* is one of the best-studied models of AHL-mediated QS. In this species, two separate autoinducer synthase/transcriptional regulator pairs, LasRI and RhlRI, modulate the expression of several genes, including many virulence factors, in response to increasing concentrations of the specific signaling molecules oxo-C_{12}-HSL and C_4-HSL (Pesci et al., in *Cell-cell signaling in bacteria*, eds., 1999; Van Delden et al., *Emerg. Infect. Dis.* **4,** 551-560, 1998).

P. aeruginosa also produces a cell-to-cell signal distinct from AHLs: 3,4-dihydroxy-2-heptylquinoline, called the PQS signal (Pesci et al., *Proc. Natl. Acad. Sci. USA* **96,** 11229-11234, 1999). PQS serves as a signaling molecule regulating the expression of a subset of genes belonging to the QS regulon, including the *phz* and *hcn* operons. PQS functions in the QS hierarchy by linking a regulatory cascade between the *las* and the *rhl* systems (McKnight et al., *J. Bacteriol.* **182,** 2702-2708, 2000). That maximal PQS production occurs at the end of the exponential growth phase (Lépine et al., *Biochim. Biophys. Acta* **1622,** 36-40, 2003) supports the hypothesis that PQS acts as a secondary regulatory signal for a subset of QS-controlled genes. Although PQS has no antibiotic activity (Pesci et al., *Proc. Natl. Acad. Sci. USA* **96,** 11229-11234, 1999), it belongs to a family of poorly characterized antimicrobial *P. aeruginosa* products, the 'pyo' compounds, originally described in 1945, which are derivatives of 4-hydroxy-2-alkylquinolines (HAQs) (Hays et al., *J. Biol. Chem.* **159,** 725-750, 1945; Wells, *J. Biol. Chem.* **196,** 331-340, 1952). The QS-associated *P. aeruginosa* transcriptional regulator, MvfR, is also required for the production of several secreted compounds, including virulence factors and PQS (Cao et al., *Proc. Natl. Acad. Sci. U S A* **98,** 14613-8, 2001; Rahme et al., *Proc. Natl. Acad. Sci. USA* **94,** 13245-13250, 1997). MvfR controls the synthesis of anthranilic acid

(AA), a PQS precursor (Calfee et al., *Proc. Natl. Acad. Sci. USA* **98**, 11633-11637, 2001), by positively regulating the transcription of *phnAB*, which encodes an anthranilate synthase (Cao et al., *Proc. Natl. Acad. Sci. USA* **98**, 14613-8, 2001).

This pathway represents a candidate target for the pharmacological intervention of *P. aeruginosa* mediated infections.

Summary of the Invention

The present invention provides screening methods to identify compounds useful (e.g., a peptide, polypeptide, synthetic organic molecule, naturally occurring organic molecule, nucleic acid molecule, or component thereof) for the treatment, prevention, or reduction of pathogenic infections caused, for example, by *Pseudomonas aeruginosa*. Using such agents as lead compounds, for example, the present screening methods also allow the identification of further novel, specific agents that function to treat, reduce, or prevent pathogenic infections.

According to one approach, candidate compounds are added at varying concentrations to the culture medium of pathogenic cells (any pathogenic cell, such as those that infect mammals (e.g., *Pseudomonas aeruginosa* such as PA14 or PAO1) or plants) after which the production of an HAQ molecule, HHQ molecule, derivatives or precursors thereof is measured using any standard method known in the art or described herein. For example, the production of the HAQ molecule is determined. Exemplary HAQ molecules, HHQ molecules, derivatives thereof, or precursors thereof are provided in Fig. 5 and Fig. 2.

Alternatively, the screening methods of the invention may be used to identify candidate compounds to decrease the production of an HAQ molecule, an HHQ molecule, a derivative thereof, or a precursor thereof (thereby decreasing the production of PQS, in turn reducing the virulence of a pathogenic cell) by their ability to reduce, treat, or prevent a pathogenic infection. Such reduction is desirably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% relative to an untreated control.

Alternatively, the screening method may involve contacting a population of pathogenic cells with a candidate compound, culturing the population of cells for a predetermined amount of time, and collecting the supernatant from the population of cells. The collected supernatant is next placed on a second population of cells

expressing a PqsH protein (or a chemical derivative thereof) after which the production of PQS from this second population is measured by any method known in the art. If the candidate compound reduces the production of PQS in this population of cells relative to a control population that has been contacted with supernatant collected from pathogenic cells cultured in the absence of a candidate compound, this candidate compound is identified as being useful for treating, preventing, or reducing a pathogenic infection by virtue of its ability to interfere with the HHQ biosynthesis pathway. The PqsH protein used in this assay may be encoded by a nucleic acid of SEQ ID NO: 6 or by a nucleic acid molecule that binds under stringent conditions to SEQ ID NO: 6 or a sequence complementary thereto. Alternatively, the PqsH protein is substantially identical to the amino acid sequence of SEQ ID NO: 13. Pathogenic cells may either endogenously express PqsH or may alternatively be genetically engineered by any standard technique known in the art (e.g., transfection and viral infection) to overexpress PqsH. The effect of a candidate compound on the production of PQS may be tested by radioactive and non-radiaoctive binding assays, competition assays, and signaling assays.

Ultimately, the screening assay of the invention may be carried out, for example, in a cell-free system. If desired, one of the Pqs proteins, HAQ molecules, HHQ molecules, a derivative or precursor thereof, or the candidate compound may be immobilized on a support or may have a detectable group.

Alternatively, or in addition, candidate compounds may be screened for those which specifically bind to and thereby reduce HAQ or HHQ activity or levels. The efficacy of such a candidate compound is dependent upon its ability to interact with such molecules. Such an interaction can be readily assayed using any number of standard binding techniques and functional assays (e.g., Ausubel et al., *Current Protocols in Molecular Biology*, 2004, John Wiley and Sons). For example, a candidate compound may be tested *in vitro* for interaction and binding with an HAQ molecule, HHQ molecule, a precursor or derivative thereof, and its ability to reduce HAQ or HHQ activity or levels may be assayed by any standard assays described in the art or those described herein.

Alternatively, the invention also provides a method of identifying a candidate compound by contacting a candidate compound, a Pqs protein (e.g., pqsA, pqsB, pqsC, pqsD, pqsE, pqsH, and pqsL protein), and an HAQ or HHQ molecule (or a

derivative/precursor thereof) capable of binding the Pqs protein under conditions that allow binding. If HHQ is used in this method instead of an HAQ molecule, a PqsH protein is used. Binding of this Pqs protein to the HAQ molecule or HHQ molecule (or a derivative/precursor thereof) is next measured such that a decrease in binding effected by the candidate compound identifies a candidate compound useful for treating, reducing, or preventing a pathogenic infection. The Pqs protein may contain an amino acid sequence substantially identical to any one of SEQ ID NOs: 8-14, or may alternatively be encoded by a nucleic acid molecule that is substantially identical to or that hybridizes under stringent conditions to any one of SEQ ID NOs: 1-7.

Alternatively, the invention also provides a method of identifying a candidate compound by contacting a candidate compound, an MvfR protein, a nucleic acid sequence substantially identical to the nucleic acid of SEQ ID NO:15 or with a nucleic acid molecule that binds under stringent conditions to SEQ ID NO:15 or a sequence complementary thereto or fragment thereof (e.g., the *lysR-box* sequence). Binding of MvfR to this nucleic acid is next measured such that a decrease in binding effected by the candidate compound identifies a candidate compound useful for treating, reducing, or preventing a pathogenic infection. The MvfR protein may contain an amino acid sequence substantially identical to any one of SEQ ID NO:17, or may alternatively be encoded by a nucleic acid molecule that is substantially identical to or that hybridizes under stringent conditions to any one of SEQ ID NO:16.

In still another embodiment, the invention also provides a method of identifying a candidate compound by contacting a candidate compound, a mutant *pqs* strain (e.g. *pqsA* mutant) strain of a cell (for example, any pathogenic cell, such as those that infect mammals (e.g., *Pseudomonas aeruginosa* such as PA14 or PAO1) or plants) containing a *pqs*-reporter (e.g., a *pqsA-LacZ* reporter) and a PQS molecule. The output level of the reporter construct is next measured such that a decrease in output effected by the candidate compound identifies a candidate compound useful for treating, reducing, or preventing a pathogenic infection.

In addition, the invention also provides a method of identifying a candidate compound by contacting a candidate compound, a nucleic acid of SEQ ID NO:15 or by contacting a nucleic acid molecule that binds under stringent conditions to SEQ ID NO:15 or a sequence complementary thereto or fragment thereof (e.g. a fragment

containing a *lysR-box*). The binding of this nucleic acid to the candidate compound identifies a candidate compound useful for treating, reducing, or preventing a pathogenic infection.

Screening for new inhibitors and optimization of lead compounds may be assessed, for example, by assessing their ability to function as anti-pathogenic agents (e.g., as described herein). Compounds isolated by this approach may also be used, for example, as therapeutics to treat, reduce, or prevent pathogenic infections. Compounds which are identified as binding to HHQ or HAQ (or a derivative/precursor thereof) with an affinity constant less than or equal to 10 mM are considered particularly useful in the invention.

Ultimately, the candidate compounds identified by the present screening methods may be used to treat, prevent, or reduce pathogenic infections in plants and mammals caused, for example, by *Pseudomonas aeruginosa.*

As used herein, by "Pqs protein" is meant any polypeptide that exhibits an activity common to its related, naturally occurring Pqs polypeptide. According to this invention, such proteins include any of the Pqs proteins, including PqsA, PqsB, PqsC, PqsD, PqsE, PqsH, and PqsL. The Pqs protein of the invention may participate in bacterial quorum sensing, a process used by bacteria to coordinate their population behavior through the action of extracellular signal molecules. The naturally occurring PqsA-E enzymes typically participate in this process by catalyzing the biosynthesis of the extracellular signal molecules, 4-hydroxy-2-alkylquinolines (HAQs). PqsH protein is typically involved in the production of PQS from HHQ. Such activities are shown in Fig. 5. Desirably, the Pqs protein of the invention is associated with such activity and in turn, increases pathogenic virulence.

Accordingly, the Pqs protein of the invention is substantially identical to any of the naturally occurring Pqs proteins, including PqsA, PqsB, PqsC, PqsD, PqsE, PqsH, and PqsL. Thus, the Pqs protein may be at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100% identical to any of the amino acid sequence of SEQ ID NOs: 8-14. Other exemplary Pqs proteins may be found in Genbank Accession Numbers ZP_00138572, ZP_00138573, ZP_00138574, ZP_00138575, ZP_00138576, ZP_00135896, and ZP_00137677. Alternatively, the Pqs protein is encoded by a nucleic acid molecule that is at least 5%, 10%, 20%, 30%, 40%, 50%,

60%, 70%, 80%, 90%, 95%, 100% identical to any one of the nucleic acid sequence of SEQ ID NOs: 1-7 or by a nucleic acid molecule that hybridizes under stringent conditions to any one of the nucleic acids of SEQ ID NOs: 1-7 or the complementary sequence thereto. Preferably, the Pqs protein increases the biosynthesis of HAQs, HHQ, or PQS or alternatively, increases pathogenic virulence by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more than 100% above control levels as measured by any standard method known in the art.

By "reduce the level or activity of Pqs protein" is meant to reduce the expression level or the biological activity of Pqs relative to the expression level or biological activity of Pqs in an untreated control. According to this invention, such level or activity is modulated by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or even greater than 100%, relative to an untreated control. Desirably, pathogenic infections are treated, prevented, or reduced if the biological activity or level of Pqs protein is reduced in a mammal or plant.

By "reduce the production of an HAQ molecule, HHQ molecule, a derivative thereof, or a precursor thereof" is meant to reduce the level or the biological activity of such HAQ molecule, HHQ molecule, (or a derivative/precursor thereof) relative to the expression level or biological activity of the corresponding molecules in an untreated control. According to this invention, such level or activity is reduced by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or even greater than 100%, relative to an untreated control as measured by any method known in the art or described herein. For example, the level of HAQ may be assayed by measuring HAQ antimicrocibial activity. HHQ levels may be determined by contacting a test sample with a population of cells expressing a PqsH protein and detecting the production level of PQS that results from such contacting. Alternatively, such reduction may also be determined by assaying the virulence of a pathogenic cell. Accordingly, a useful candidate compound reduces the virulence of a pathogenic cell. Desirably, pathogenic infections are treated, prevented, or reduced if the production or level of an HAQ or HHQ (or a derivative/precursor thereof) is reduced in a mammal or plant by interfering with PQS production, in turn reducing the virulence of pathogenic cells. Such exemplary molecules are shown in Fig. 2 and 5.

By a "candidate compound" is meant a chemical, be it naturally-occurring or artificially-derived. Candidate compounds may include, for example, peptides, polypeptides, synthetic organic molecules, naturally occurring organic molecules, nucleic acid molecules, peptide nucleic acid molecules, and components and derivatives thereof.

By "nucleic acid molecule" is meant multiple nucleotides, each of which contains a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G). As used herein, the term refers to oligoribonucleotides as well as oligodeoxyribonucleotides. The nucleic acid molecules of the invention also include polynucleosides (i.e. a polynucleotide lacking the phosphate) and any other organic base-containing polymer. Nucleic acid molecules may be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but if desired, may be synthetic (e.g. produced by oligonucleotide synthesis).

By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 25% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 30%, 40%, 50%, 60%, 70%, more preferably 80%, 81%, 82%, 83%, 84%, 85% identical, and most preferably 90%, 92%, 94%, 95%, 96%, 97%, 98%, or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine,

alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

By "pathogenic infection" is meant any infection caused by the presence of a microbial agent, such as a bacterium. According to this invention, pathogens may infect a mammal (e.g., human) or a plant. Bacterial infections may be caused by Gram-positive and Gram-negative agents (e.g., *Pseudomonas aeruginosa*), that may or may not display antibiotic resistance. Pathogenic infections may or may not be symptomatic.

By "treating, reducing, or preventing a pathogenic infection" is meant reducing such infection before or after it has occurred. As compared with an equivalent untreated control, such reduction or degree of prevention is at least 5%, 10%, 20%, 40%, 50%, 60%, 80%, 90%, 95%, or 100% as measured by any standard technique that detects the level of the pathogen. Typically, a patient who is being treated for a pathogenic infection is one who a medical practitioner has diagnosed as having such a condition. Diagnosis may be performed by any suitable means known in the art or described herein. Typically, infections are diagnosed by the evaluation of symptoms, or alternatively, by the detection of the pathogen in a biological specimen in a culture assay. Similarly, a reduction in a pathogenic infection may be measured by monitoring pathogenic symptoms in a patient exposed to a candidate compound or extract, a decrease in the level of symptoms relative to the level of pathogenic symptoms in a patient not exposed to the compound indicating compound-mediated inhibition of the pathogen.

A patient in whom the development of a pathogenic infection is being prevented may or may not have received such a diagnosis. One skilled in the art will understand that these patients may have been subjected to the same standard tests as described above or may have been identified, without examination, as one at high risk due to the presence of one or more risk factors (e.g., contact with infected patient).

By "an effective amount" is meant an amount of a compound, alone or in a combination, required to treat, reduce, or prevent a pathogenic infection. The effective amount of active compound(s) varies depending upon the route of

administration, age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen.

The term "pharmaceutical composition" is meant any composition, which contains at least one therapeutically or biologically active agent and is suitable for administration to the patient. Any of these formulations can be prepared by well-known and accepted methods of the art. See, for example, Remington: The Science and Practice of Pharmacy, 20th edition, (ed. AR Gennaro), Mack Publishing Co., Easton, PA, 2000.

By "isolated nucleic acid molecule" is meant a nucleic acid (e.g., a DNA) that is free of the genes which, in the naturally occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule which is transcribed from a DNA molecule, as well as a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By a "substantially pure polypeptide" is meant a polypeptide of the invention that has been separated from components which naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, a polypeptide of the invention. A substantially pure polypeptide of the invention may be obtained, for example, by extraction from a natural source (for example, a pathogen); by expression of a recombinant nucleic acid encoding such a polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "pathogenic virulence factor" is meant a cellular component (e.g., a protein such as a transcription factor, as well as the gene which encodes such a protein) without which the pathogen is incapable of causing disease or infection in a eukaryotic host organism.

By "antisense" is meant a nucleic acid, regardless of length, that is complementary to a coding strand or mRNA of the invention. In some embodiments, the antisense molecule inhibits the expression of only one nucleic acid, and in other embodiments, the antisense molecule inhibits the expression of more than one nucleic acid. Desirably, the antisense nucleic acid decreases the expression or biological activity of a nucleic acid or protein of the invention by at least 20, 40, 50, 60, 70, 80, 90, 95, or 100%. An antisense molecule can be introduced, e.g., to an individual cell or to whole animals, for example, it may be introduced systemically via the bloodstream. Desirably, a region of the antisense nucleic acid or the entire antisense nucleic acid is at least 70, 80, 90, 95, 98, or 100% complementary to a coding sequence, regulatory region (5' or 3' untranslated region), or an mRNA of interest. Desirably, the region of complementarity includes at least 5, 10, 20, 30, 50, 75,100, 200, 500, 1000, 2000, or 5000 nucleotides or includes all of the nucleotides in the antisense nucleic acid.

In some embodiments, the antisense molecule is less than 200, 150, 100, 75, 50, or 25 nucleotides in length. In other embodiments, the antisense molecule is less than 50,000; 10,000; 5,000; or 2,000 nucleotides in length. In certain embodiments, the antisense molecule is at least 200, 300, 500, 1000, or 5000 nucleotides in length. In some embodiments, the number of nucleotides in the antisense molecule is contained in one of the following ranges: 5-15 nucleotides, 16-20 nucleotides, 21-25 nucleotides, 26-35 nucleotides, 36-45 nucleotides, 46-60 nucleotides, 61-80 nucleotides, 81-100 nucleotides, 101-150 nucleotides, or 151-200 nucleotides, inclusive. In addition, the antisense molecule may contain a sequence that is less than a full-length sequence or may contain a full-length sequence.

The invention provides a number of targets that are useful for the development of drugs that specifically block the pathogenicity of a microbe, for example, *Pseudomonas aeruginosa* PA14. In addition, the methods of the invention provide a facile means to identify compounds that are safe for use in eukaryotic host organisms (i.e., compounds which do not adversely affect the normal development and

physiology of the organism), and efficacious against pathogenic microbes (i.e., by suppressing the virulence of a pathogen). In addition, the methods of the invention provide a route for analyzing virtually any number of compounds for an anti-virulence effect with high-volume throughput, high sensitivity, and low complexity. The methods are also relatively inexpensive to perform and enable the analysis of small quantities of active substances found in either purified or crude extract form.

Brief Description of the Figures

Fig. 1 shows LC/MS analysis of PA14 and *mvfR* mutant culture supernatants. MS chromatograms of PA14 (upper trace) and the *mvfR* mutant (lower trace). The numbers above the peaks represent the m/z values of the most intense ions. Intensities are normalized to the most abundant ion in the upper trace

Fig. 2 shows chemical structures of five distinct series of HAQ compounds isolated from the PA14 culture supernatant. Detailed MS analysis of the peaks in Fig. 1. R, alkyl side chain length.

Fig. 3 shows the expression profiles of *pqsA* and *phnA* in PA14 versus the *mvfR* mutant, using the GeneChip® *P. aeruginosa* array. (●), PA14; (□), the *mvfR* mutant. Signal intensity values calculated by dCHIP software. OD, optical density at 600 nm of the cultures when harvested.

Fig. 4 shows HAQ production kinetics in PA14 versus the isogenic *pqsE* mutant. Bacteria were grown in LB at 37°C and their extracellular HAQ concentrations were analyzed by MS at regular time intervals. Solid symbols, PA14; Open symbols, *pqsE* mutant; (■,□), Optical density of the culture [OD_{600}]; (●,○), PQS; (▲,△), HQNO; (◆,◇), HHQ.

Fig. 5 is a schematic diagram showing the HAQ biosynthetic pathway in *P. aeruginosa*. The sequence of synthesis was determined by supplementing cultures of PA14 and various *pqs/mvfR* mutants with deuterium-labelled intermediates. Bracketed structures are hypothetical.

Fig. 6 is a schematic diagram showing HHQ/PQS cell-to-cell communication model. (1) HHQ is synthesized and released by bacterial cells; (2) extracellular HHQ is taken up by adjacent bacteria and converted into PQS, possibly in the periplasm; (3) PQS is released to act as a signaling molecule for other cells; and (4) PQS activates target gene expression, such as the *phz1* operon. Note that both HHQ

availability and PqsH activity determine the final PQS concentration. In the experimental paradigm used to test the model (see text), cell [A] and cell [B] were an *mvfR* mutant and a *lasR* mutant, respectively. In the case of a wild type population, both the [A] and [B] cells are producing HHQ and PQS.

Fig. 7 shows Pyocyanin production in a mixed-mutant culture illustrates the HAQ cell-to-cell communication pathway. Culture suspensions of the *mvfR* mutant, the *lasR* mutant, or a 1:1 mixture of both, were spotted onto an LB plate and incubated for 18 hrs at 37°C. Only the mixed culture produces detectable amounts of pyocyanin, presumably from the *lasR*⁻ cells, as *mvfR*⁻ cells are deficient for *phz1* operon expression. This result demonstrates that HHQ, produced by the *lasR*⁻ cells, is released and taken up by the *mvfR*⁻ cells and converted into PQS. This PQS signal is then released by the *mvfR*⁻ cells and taken up by the *lasR*⁻ cells, where it signals *phz1* expression and pyocyanin production.

Fig. 8. shows the MS spectra of HHQ, PQS and HQNO in PA14 culture supernatant grown in the presence or absence of 3,4,5,6-tetradeutero-anthranilic acid. The six traces from the top to the bottom of figure respectively correspond to (1) unlabeled HHQ; (2) deuterated HHQ; (3) unlabeled PQS; (4) deuterated PQS; and (5) unlabeled HQNQ; and (6) deuterated HQNQ.

Fig. 9 shows expression of the *pqsABCDE* and *phnAB* genes in whole-genome transcriptome profiles of PA14 and the *mvf*R mutant using the *P. aeruginosa* GeneChip® array. (●), PA14; (□), *mvfR* mutant. OD, optical density of the harvested culture samples at 600 nm. Signal intensity values were calculated using dCHIP.

Fig. 10 shows an assay of HAQ antimicrobial activity. *Bacillus subtilis* growth on well plates was scored for inhibition by culture extracts of PA14 and the *mvfR*, *pqsA*, and *pqsE* mutant strains (left plate), and by purified PQS, HHQ and HQNO, versus PA14 extract (right plate).

Fig. 11 shows the nucleic acid sequences and amino acid sequences of PqsA-E, H, and L and mvfR, as well as the promoter region of the pqsA-E operon.

Fig. 12 is a table showing the percent relative concentration of extracellular compounds in culture supernatants of PA14 mutant strains versus the wild type PA14 strain. Cells were cultivated in LB medium for 11 hrs at 37°C to a final $OD_{600} = 4$. Data are averages of triplicate experiments ± SD. HHQ, 4-hydroxy-2-

heptylquinoline; PQS, *Pseudomonas* quinolone signal (3,4-dihydroxy-2-heptylquinoline); HNQ, 4-hydroxy-2-nonylquinoline; diHNQ, 3,4-dihydroxy-2-nonylquinoline; AA, anthranilic acid; NT, not tested; *mvfR* compl. is the mutant strain complemented with pDN18*mvfR*.

Fig. 13 is a table showing the concentration of PQS (μg/ml) and *phz1* gene expression in a *lasR* mutant culture and a 1:1 *lasR* mutant: *mvfR* mutant culture. Cultures were assayed at 8 hr sampling time, corresponding to OD_{600} 4.3 to 4.5. *Ratios have been corrected by taking into account that the mixed culture contains 50 % less *lasR* mutant cells than the *lasR* culture. *mvfR* mutant cells do not produce PQS. †The *lasR* mutant carries a *phzABC-lacZ* fusion. Data correspond to averages of triplicates ± standard deviation; MU: Miller units.

Fig. 14 is a table showing the effect of HHQ addition on *phz1* gene expression in PA14 and *lasR*⁻ cultures. Cultures were assayed at 8 hr, corresponding to OD_{600} 4.3 to 4.5. The bacteria carry a *phzABC-lacZ* fusion. Data correspond to averages of triplicates ± standard deviation; MU: Miller units.

Fig. 15 shows *PqsA-lacZ* induction by PQS, anthranilic acid, and cell supernatant extract. Compounds were added at the onset of culture at 10 mg/L final concentration. Activity is reported as Miller units (MU), corrected for the OD of the cultures at 600 nm.

Fig. 16 shows analysis of mutants of the *pqsA* regulatory region. (A) *las*-box and *lysR* box plasmid mutants based on the wild-type *pqsA'-lacZ* reporter construct, pGX5; (B) PA14 expression of the *pqsA-lacZ* constucts; (C) PA14 expression of wild type (pGX7) and *lysR* box mutants; (D) Expression of the *pqsA'-lacZ* transcriptional reporter pGX7 in PA14, and in an isogenic *mvfR* mutant.

Detailed Description

In general, the present invention is based on our discovery that the pathogenic Pqs proteins mediate the biosynthesis of of extracellular signalling molecules involved in bacterial quorum sensing. Based on our results, the invention provides screening methods for identifying candidate compounds useful for the treatment, reduction, or prevention of pathogenic infections. Also disclosed are methods or treating, reducing, or preventing pathogenic infections, caused, for example, by *Pseudomonas aeruginosa*.

Bacterial communities utilize 'Quorum Sensing' (QS) to coordinate their population behavior through the action of extracellular signal molecules, such as the *N*-acyl-L-homoserine lactones (AHLs). The versatile and ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* is a well-studied model for AHL-mediated QS. This species also produces an intercellular signal distinct from AHLs, 3,4-dihydroxy-2-heptylquinoline (PQS), which belongs to a family of poorly characterized 4-hydroxy-2-alkylquinolines (HAQs) which are associated with antimicrobial activity.

Here we use LC/MS, genetics, and whole-genome expression to investigate the structure, biosynthesis, regulation, and activity of HAQs. We first demonstrate that the MvfR transcriptional regulator controls *pqsA-E* expression. Our results show that the *pqsA-E* operon encodes enzymes that direct the biosynthesis of five classes of HAQs, including molecules that function as antibiotics and cytochrome inhibitors and, significantly, as intercellular communication molecules. We have also found that anthranilic acid, the product of the PhnAB synthase, is the primary precursor of HAQs; and that the HAQ congener 4-hydroxy-2-heptylquinoline (HHQ) is the direct precursor of the PQS signaling molecule, which is itself a message molecule involved in cell-to-cell communication. While *phnAB* and *pqsA-E* are positively regulated by the virulence-associated transcription factor MvfR, which is also required for the expression of several QS-regulated genes, the conversion of HHQ to PQS is instead controlled by LasR. Our results further reveal that HHQ is itself both released from, and taken up by bacterial cells, where it is converted into PQS, which may subsequently function as a messenger molecule in a cell-to-cell communication pathway. HAQ signaling represents a potential target for the pharmacological intervention of *P. aeruginosa*-mediated infections.

Screening Assays

The present invention provides screening methods to identify compounds useful (e.g., a peptide, polypeptide, synthetic organic molecule, naturally occurring organic molecule, nucleic acid molecule, or component thereof) for the treatment, prevention, or reduction of pathogenic infections. Using such agents as lead compounds, for example, the present screening methods also allow the identification of further novel, specific agents that function to treat, reduce, or prevent pathogenic infections. The method of screening may involve high-throughput techniques. A number of methods are available for carrying out such screening assays.

According to one approach, candidate compounds are added at varying concentrations to the culture medium of pathogenic cells (any pathogenic cell, such as those that infect mammals (e.g., *Pseudomonas aeruginosa* such as PA14 or PAO1) or plants) after which the production of an HAQ molecule, HHQ molecule, or a derivative or precursor thereof is measured using any standard method known in the art or described herein. For example, the production of an HAQ molecule may be determined by measuring the antimicrobial activity of the HAQ molecule. Exemplary HAQ molecules, HHQ molecules, or precursors or derivatives thereof are provided in Fig. 5 and Fig. 2.

Alternatively, the screening methods of the invention may be used to identify candidate compounds to decrease the production of an HAQ molecule or an HHQ molecule (thereby decreasing the production of PQS, in turn reducing the virulence of a pathogenic cell) by their ability to reduce, treat, or prevent a pathogenic infection. Such reduction is desirably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% relative to an untreated control. The virulence of a pathogenic cell may be measured using the cell-based methods or *in vivo* methods herein.

Alternatively, the screening method may involve contacting a population of pathogenic cells with a candidate compound, culturing the population of cells for a predetermined amount of time, and collecting the supernatant from the population of cells. A useful candidate compound would inhibit any step of the HHQ biosynthesis pathway shown in Fig. 5 or Fig. 2, such that the production of HHQ would be reduced. The collected supernatant is next placed on a second population of cells expressing a PqsH protein (or a chemical derivative thereof) after which the production of PQS from this second population is measured by any method known in the art. If the candidate compound reduces the production of PQS in this population of cells relative to a control population that has been contacted with supernatant collected from pathogenic cells cultured in the absence of a candidate compound, this candidate compound is identified as being useful for treating, preventing, or reducing a pathogenic infection by virtue of its ability to interfere with the HHQ biosynthesis pathway. The PqsH protein used in this assay may be encoded by a nucleic acid of SEQ ID NO: 6 or by a nucleic acid molecule that binds under stringent conditions to SEQ ID NO: 6 or a sequence complementary thereto. Alternatively, the PqsH protein is substantially identical to the amino acid sequence of SEQ ID NO: 13. Pathogenic

cells may either endogenously express PqsH or may alternatively be genetically engineered by any standard technique known in the art (e.g., transfection and viral infection) to overexpress PqsH. The effect of a candidate compound on the production of PQS may be tested by radioactive and non-radiaoctive binding assays, competition assays, and signaling assays.

Ultimately, the screening assay of the invention may be carried out, for example, in a cell-free system. If desired, one of the Pqs proteins, HAQ molecules, HHQ molecules, or the candidate compound may be immobilized on a support as described above or may have a detectable group.

Alternatively, or in addition, candidate compounds may be screened for those which specifically bind to and thereby reduce HAQ or HHQ activity or levels. The efficacy of such a candidate compound is dependent upon its ability to interact with such molecules. Such an interaction can be readily assayed using any number of standard binding techniques and functional assays (e.g., Ausubel et al., *Current Protocols in Molecular Biology*, 2004, John Wiley and Sons). For example, a candidate compound may be tested *in vitro* for interaction and binding with an HAQ molecule or HHQ molecule or derivative or precursor thereof and its ability to reduce HAQ or HHQ activity or levels may be assayed by any standard assays (e.g., those described herein).

In one particular example, a candidate compound that binds to HHQ may be identified using a chromatography-based technique. For example, an HHQ molecule may be produced by standard techniques (e.g., those described above) and may be immobilized on a column. Alternatively, the naturally-occurring HHQ molecule may be immobilized on a column. A solution of candidate compounds is then passed through the column, and a compound specific for HHQ is identified on the basis of its ability to bind to HHQ and be immobilized on the column. To isolate the compound, the column is washed to remove non-specifically bound molecules, and the compound of interest is then released from the column and collected. Compounds isolated by this method (or any other appropriate method) may, if desired, be further purified (e.g., by high performance liquid chromatography).

Alternatively, the invention also provides a method of identifying a candidate compound by contacting a candidate compound, a Pqs protein (e.g., pqsA, pqsB, pqsC, pqsD, pqsE, pqsH, and pqsL protein), and an HAQ or HHQ molecule capable

of binding the Pqs protein under conditions that allow binding. If HHQ is used in this method instead of an HAQ molecule, a PqsH protein is used. Binding of this Pqs protein to the HAQ molecule or HHQ molecule is next measured such that a decrease in binding effected by the candidate compound identifies a candidate compound useful for treating, reducing, or preventing a pathogenic infection. The Pqs protein may contain an amino acid sequence substantially identical to any one of SEQ ID NOs: 8-14, or may alternatively be encoded by a nucleic acid molecule that is substantially identical to or that hybridizes under stringent conditions to any one of SEQ ID NOs:1-7.

Alternatively, the invention also provides a method of identifying a candidate compound by contacting a candidate compound, an MvfR protein, a nucleic acid sequence substantially identical to the nucleic acid of SEQ ID NO:15 or with a nucleic acid molecule that binds under stringent conditions to SEQ ID NO:15 or a sequence complementary thereto or fragment thereof (e.g. the *lysR-box* sequence). Binding of MvfR to this nucleic acid is next measured such that a decrease in binding effected by the candidate compound identifies a candidate compound useful for treating, reducing, or preventing a pathogenic infection. The MvfR protein may contain an amino acid sequence substantially identical to any one of SEQ ID NO:17, or may alternatively be encoded by a nucleic acid molecule that is substantially identical to or that hybridizes under stringent conditions to any one of SEQ ID NO:16.

Alternatively, the invention also provides a method of identifying a candidate compound by contacting a candidate compound, a cell (for example, a cell such as a mutant *pqs* strain (e.g., a *pqsA* mutant strain) of a pathogenic cell (any pathogenic cell, such as those that infect mammals (e.g., *Pseudomonas aeruginosa* such as PA14 or PAO1) or plants)) containing a *pqs*-reporter (e.g., a *pqsA-LacZ* reporter), and a PQS molecule. The output level of the reporter construct is next measured such that a decrease in output effected by the candidate compound identifies a candidate compound useful for treating, reducing, or preventing a pathogenic infection.

Alternatively, the invention also provides a method of identifying a candidate compound by contacting a candidate compound, a nucleic acid of SEQ ID NO:15 or by a nucleic acid molecule that binds under stringent conditions to SEQ ID NO:15 or a sequence complementary thereto or fragment thereof (e.g., a fragment containing a

lysR-box). The binding of this nucleic acid to the candidate compound identifies a candidate compound useful for treating, reducing, or preventing a pathogenic infection.

Screening for new inhibitors and optimization of lead compounds may be assessed, for example, by assessing their ability to function as anti-pathogenic agents (e.g., as described herein). Compounds isolated by this approach may also be used, for example, as therapeutics to treat, reduce, or prevent pathogenic infections. Compounds which are identified as binding to HHQ or HAQ with an affinity constant less than or equal to 10 mM are considered particularly useful in the invention.

Ultimately, the anti-pathogenic efficacy of any of the candidate compounds identified by the present screening methods may be tested using any of the pathogenicity models described herein or known in the art.

Potential therapeutic agents include organic molecules, peptides, peptide mimetics, polypeptides, and antibodies. Potential anti-pathogenic agents also include small molecules that bind to and occupy the binding site of Pqs polypeptides thereby preventing binding to cellular binding molecules, such that normal biological activity is reduced, in turn reducing HHQ and PQS production and pathogenic virulence. Other potential anti-pathogenic agents may also include antisense molecules.

Test compounds and extracts

In general, compounds capable of treating, reducing, or preventing pathogenic infections are identified from large libraries of both natural products or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are

commercially available. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are also commercially available. In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their anti-hypertensive activity should be employed whenever possible.

When a crude extract is found to have an anti-pathogenic activity, or a binding activity, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having anti-pathogenic activity. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for the treatment of pain are chemically modified according to methods known in the art.

Results

HAQ identification: *mvfR* is required for the production of five distinct series of HAQs from the common precursor anthranilic acid

Calfee *et al.* (*Proc. Natl. Acad. Sci. USA* **98,** 11633-11637, 2001) recently reported that ^{14}C-labelled AA is incorporated into PQS, but that this PQS represents only 12 % of the newly synthesized compounds, indicating that the ethyl acetate extract contains additional AA-derived molecules. Because HHQ biosynthesis proceeds from the coupling of AA and an α-keto fatty acid (Ritter et al., *Eur. J. Biochem.* **18,** 391-400, 1971), we hypothesized that these unidentified AA-derived molecules might correspond to HAQs related to PQS and HHQ.

To this end, we fed AA or deuterated AA (AA-d_4) to PA14 cultures, and analyzed the culture supernatants using LC/MS (Lépine et al., *Biochim. Biophys. Acta* **1622,** 36-40, 2003). The resulting chromatograms exhibit several peaks in the vicinity

of PQS and the mass spectra of these compounds all show the addition of 4 Da in the cultures fed AA-d_4, demonstrating that AA is their common precursor (Fig. 8). Since we have previously shown that PQS production is abrogated in an *mvfR* mutant (Cao et al., *Proc Natl. Acad. Sci. USA* **98,** 14613-8, 2001), we investigated the synthesis of these compounds in this mutant. Fig. 1 shows that all the deuterium-labeled peaks are absent from the *mvfR* mutant culture supernatant; with the only residual peaks found at HAQ retention times corresponding to two conformers of the siderophore pyochelin (Rinehart et al., *J. Org. Chem.* **60,** 2786-2791, 1995), which give M+H ions at m/z 325 and are structurally unrelated to HAQs.

The mass spectra of these labeled peaks show that they correspond to five distinct series of HAQs (Fig. 2). All these congeners share the common basic 4-hydroxyquinoline structure of series A with an additional hydroxyl at the 3-position, as in series B, or with an *N*-oxide group as in series C and E. Within each series, the 2-position alkyl chain varies in length. Also, the series D and E alkyl side chain is unsaturated. The most abundant congeners contain an odd carbon number alkyl chain, with seven or nine carbons preponderant.

The HAQ congeners include both previously identified and novel compounds. The C_7 (HHQ) and C_9 (HNQ) congeners are shown in Fig. 2 (also see Wells, *J. Biol. Chem.* **196,** 331-340, 1952), while the structures of the other series A congeners were later determined using GC/MS (Taylor et al., *J. Chromatogr. B Biomed. Appl.* **664,** 458-62, 1995). In contrast, the only reported series B congener is 3,4-dihydroxy-2-heptylquinoline, first isolated in 1959 (Takeda, *Hakko Koyaku Zasshi* **37,** 59-63, 1959), and later fully characterized and named PQS (Pesci et al., *Proc. Natl. Acad. Sci. USA* **96,** 11229-11234, 1999). For series C, the C_7, C_9, C_8 and C_{11} congeners have been reported (Cornforth et al., *Biochem. J.* **63,** 124-130, 1956; Luckner et al., *Tetrahedron Letters* **12,** 741-744, 1965), and are further discussed below, while the series E and the series B PQS congeners are unique to this study. The novelty of many of our HAQs, such as the series B molecules, and the series C and E congeners, which have polar *N*-oxide functions, is because previous studies employed derivatization prior to GC/MS injection (Machan et al., *J. Antimicrob. Chemother.* **30,** 615-623, 1992; Taylor et al., *J. Chromatogr. B Biomed. Appl.* **664,** 458-62, 1995). Positive electrospray ionization mass spectrometry is better suited than GC/electron impact-MS for the detection of such relatively basic compounds.

HAQ Regulation: MvfR controls the expression of the *phnAB* and *pqsA-E* operons, which are required for HAQ synthesis

That MvfR regulates *phnAB* expression (Cao et al., *Proc. Natl. Acad. Sci. USA* **98,** 14613-8, 2001) suggests that it might also direct HAQ biosynthesis by regulating genes that encode anabolic pathway enzymes. As part of a project to identify MvfR-regulated genes, we carried out a transcriptome comparative analysis between PA14 and its isogenic *mvfR* mutant at set time points during a growth time-course, using the Affymetrix *P. aeruginosa* GeneChip® oligonucleotide array. The expression profiles of the five genes just upstream from the anthranilate synthase *phnAB* operon tightly cluster with *phnAB* expression (Fig. 3 and Fig. 9), suggesting they are co-regulated. Fig. 4 shows that the expression patterns of these seven genes correlate with the kinetic rates of HAQ production, which are maximal at the end of exponential/early stationary phase (i.e., OD_{600} 2.5) (Lépine et al., *Biochim. Biophys. Acta* **1622,** 36-40, 2003). Since *phnAB* expression is under the control of MvfR (Cao et al., *Proc. Natl. Acad. Sci. USA* **98,** 14613-8, 2001), it is not surprising that the transcription of these seven genes is almost completely abolished in the *mvfR* mutant (Fig. 3 and Fig. 9).

Fig. 12 shows that knockout inactivation of *pqsA* or *pqsB* results in the complete elimination of HAQ production and the striking accumulation of AA in culture supernatants. AA likely accumulates because these mutants fail to consume AA for HAQ synthesis, further supporting AA as the HAQ precursor. In contrast, a *pqsE* mutant produces wild-type levels of HAQ and AA (Fig. 4), in agreement with the observation that *pqsE* inactivation does not affect PQS production (Gallagher et al., *J Bacteriol* **184,** 6472-80, 2002). Genetic complementation suggests that *pqsABCDE* is a single operon (Gallagher et al., *J Bacteriol* **184,** 6472-80, 2002). Our expression profiling data corroborate the LC/MS results and further indicate that MvfR controls the transcription of the co-regulated *pqsABCDE* and *phnAB* operons.

HAQ activity: MvfR regulates HQNO antimicrobial activity against Gram-positive bacteria

Fluorescent pseudomonads, and perhaps just *P. aeruginosa*, are the only microorganisms identified to produce HAQs (Budzikiewicz, *FEMS Microbiol. Rev.* **104,** 209-228, 1993; Lépine et al., *Biochim. Biophys. Acta* **1622,** 36-40, 2003). Although their biological functions are unknown, many HAQs were initially isolated

as antibiotics (Hays et al., *J. Biol. Chem.* **159,** 725-750, 1945; Wells, *J. Biol. Chem.* **196,** 331-340, 1952). We assayed the antimicrobial activity of total organic extracts from PA14 and *mvfR*, *pqsA*, and *pqsE* mutant strains, and purified PQS, HHQ and HQNO, against the Gram-positive species *Staphylococcus aureus* and *Bacillus subtilis*. PA14 and *pqsE*⁻ extracts clearly inhibit both species, while the *pqsA*⁻ and *mvfR*⁻ extracts have low or no antibacterial activity (Fig. 10). Thus, MvfR regulates the production of antibiotics that can function in niche competition against other bacteria. Because the *pqsE* mutant and PA14 produce roughly the same levels of antimicrobial activity, these antibiotic compounds are probably HAQs, instead of other non-polar compounds whose synthesis is under PQS control.

While it has been known for many decades that *P. aeruginosa* produces low molecular weight antibiotics, later found to be HAQs (Bouchard, *Compt. rend Acad. Sci.* **108,** 713-714, 1889; Hays et al., *J. Biol. Chem.* **159,** 725-750, 1945), these compounds have been little characterized. Although the HAQ *N*-oxides (C_7-, C_9-, and C_{11}-) were originally isolated as streptomycin and dihydrostreptomycin antagonists (Sureau et al., *Ann. Inst. Pasteur Paris* **75,** 169-171, 1948; Lightbown, *J. Gen. Microbiol.* **11,** 477-492,1954; Cornforth et al., *Biochem. J.* **63,** 124-130, 1956), their mode of action remains unknown. We confirm here the specific antibacterial activity of HQNO (Fig. 10), in agreement with Machan et al., *J. Antimicrob Chemother* **30,** 615-623, 1992. This C_7 congener, which is a widely used cytochrome inhibitor (Lightbown et al., *Biochem. J.* **63,** 130-137, 1956), and also inhibits Na^+-translocating NADH-quinone oxidoreductases (Häse et al., *Microbiol Mol Biol Rev* **65,** 353-70 2001), may therefore function in nature as a virulence factor.

HAQ biosynthetic machinery: PQS is not a product of the *mvfR*-regulated synthetic pathway

The similarity of the HAQ structures, along with their co-labeling from deuterated AA, suggests they are produced via a common biosynthetic pathway. To this end, we added various known or putative labeled HAQ precursors and derivatives to cultures of PA14 or isogenic *pqs* and *mvfR* mutants, to generate the pathway in Fig. 5. Addition of HHQ-d_4 to PA14 cultures results in overproduction and labeling of PQS, indicating that HHQ is an intermediate in PQS biosynthesis. Also, as no overproduction or labeling of HQNO is detected, HHQ is not a precursor of HQNO.

By analogy, the series A compounds, which include HHQ, are the probable precursors of the corresponding series B compounds, but not the other series. To examine whether PQS is an intermediate in HAQ biosynthesis, PQS-d_4 was added to PA14 cultures. Since none of the chromatographic peaks are labeled, PQS must be an end-product in HAQ biosynthesis, or at least is not substantially converted into an extracellular compound. Also, as all HHQ-d_4 added to *pqs/mvfR* mutant cultures is completely converted into PQS, MvfR does not control the final step(s) of PQS synthesis.

We were unable to determine the precise origin(s) of the *N*-oxides (series C and E). Nevertheless, these HAQs clearly belong to the above pathway since AA-d_4 addition results in labeled *N*-oxides, and they are absent in mutants that fail to synthesize HAQs; however, as proposed in Fig. 5, *N*-oxides are probably not synthesized downstream of HHQ or PQS, because cultures supplemented with these deuterated compounds do not produce the corresponding labeled *N*-oxides. The *N*-oxides do not appear to be HAQ precursors, via simple reduction of their *N*-oxide function, since adding HQNO-d_4 to PA14 cultures neither results in decreased labeled *N*-oxide concentrations in the culture medium, nor labeling of any HAQ congeners. Because HQNO is a cytochrome inhibitor and has antimicrobial activity, it seems likely that it is actively exported, which would mask its role in HAQ biosynthesis in our assay. Nevertheless, *N*-oxides are probably the end-products of a branch pathway that is not under *mvfR* regulation. Indeed, we have shown that series A, B and D congener concentrations decrease in culture supernatants after peaking, whereas the concentrations of the *N*-oxides (series C and E) remain stable (Lépine et al., *Biochim. Biophys. Acta* **1622**, 36-40, 2003).

Overexpression of *mvfR* results in the excessive accumulation of series A compounds (the series B precursors; HHQ and HNQ in Fig. 12) in the supernatant, but leaves series B, C and E (PQS congeners and *N*-oxides) concentrations largely unaffected (Fig. 12), indicating that the series A (and D) congeners are end-products of the *mvfR*-regulated synthetic pathway. These data also support the conclusions that MvfR does not directly control PQS production, and that the branch pathways leading to series B, C, and E are saturated when the *mvfR*-regulated pathway is over-activated. Similarly, that a *lasR* mutant produces significant amounts of HAQs (Fig. 12), with the over-accumulation of series A congeners (the PQS analogue precursors), suggests

that the QS transcriptional regulator LasR controls the series A to series B conversion. This step is likely mediated by the *PqsH*-encoded FAD-dependent monoxygenase, a QS-controlled gene that is required for PQS synthesis (Gallagher et al., *J. Bacteriol.* **184,** 6472-80, 2002) and is under LasR regulation (Whiteley et al., *Proc. Natl. Acad. Sci. USA* **96,** 13904-1890, 1999).

Collectively, our results show that the series A compounds are the end-product of the *mvfR*-controlled biosynthetic pathway, and are subsequently converted into the series B PQS analogues via a *lasR*-dependent pathway, likely via the PqsH enzyme, which is not under MvfR regulation. These results suggest that the final synthesis of the active PQS signal is highly regulated and under additional controls beyond those of the primary HAQ pathway.

HHQ, the PQS precursor, functions in cell-to-cell communication

PQS is an extracellular signal that participates in the QS circuitry. Several observations suggest that HHQ also functions as an intercellular messenger: (a) it is released by bacteria; (b) its concentration rises during exponential growth phase, and then decreases during PQS production (Fig. 4); (c) it is taken up by bacterial cells, converted into PQS, and then released into the extracellular milieu, as shown in the labeling experiments; and (d) its synthetic pathway, via PqsA-D is distinct and under different regulation than that of HHQ-to-PQS conversion, via PqsH. These results collectively suggest the model depicted in Fig. 6, in which HHQ is released by cells and acts as a messenger that is subsequently converted into the PQS signal by the cells that take it up. To test this hypothesis, we compared PQS production in a *lasR* mutant culture, versus that of a mixed culture of *lasR* and *mvfR* mutant cells. Fig. 12 shows that the *lasR* mutant produces low levels of PQS and accumulates high concentrations of HHQ due to its low PqsH activity; and the *mvfR* mutant produces no PQS, as it is unable to synthesize the HHQ precursor. Thus, using these two mutants, one able to produce HHQ but unable to process it into PQS, and the other unable to produce HHQ, but able to convert it into PQS, should allow us to verify whether a *P. aeruginosa* cell can produce PQS using HHQ produced by another cell. Indeed, Fig. 13 shows that when the two mutants are grown together, PQS concentration is up to five times higher than if the cells fail to exchange the signaling information.

phz1 operon expression, which is required for the synthesis of pyocyanin, depends on both PQS signaling and *pqsE* expression. Therefore, to determine whether the PQS that is produced and released by *mvfR*⁻ cells, when they are co-cultured with the *lasR*⁻ cells, is biologically active in adjacent cells, we introduced a *phzABC-lacZ* reporter fusion into the *lasR*⁻ mutant, where *pqsE* is expressed, and compared the β-galactosidase activity with and without co-cultivation with *mvfR* mutant cells. Figure 13 shows that *phz1* operon expression is indeed upregulated in the presence of *mvfR*⁻ cells, indicating that the PQS produced by *mvfR* mutant cells is taken up by the *lasR*⁻ bacteria, where it activates the *phz1* operon. Accordingly, the mixed culture, but not the cultures of either mutant alone, also generates pyocyanin (Fig. 7). The *lasR* mutant is responsible for this pyocyanin production, because while the *mvfR* mutant "sees" PQS, it fails to express *pqsE*. Indeed, no β-galactosidase induction is obtained in a cocultivation experiment where the *phzABC-lacZ* reporter is carried by the *mvfR*⁻ versus the *lasR*⁻ cells, Although mutants were used for this demonstration, the "conversational" pathway presented in Fig. 6 may occur in wild-type cell cultures, since PA14 cells similarly perform HHQ release, uptake, and PQS conversion.

To determine whether HHQ is itself involved in signal gene regulation, independently from PQS activity, or whether it functions solely as a PQS precursor, we asked whether exogenous HHQ can stimulate the activity of a *phzABC-lacZ* reporter carried by wild-type PA14 cells, and by *lasR*⁻ mutant cells. While HHQ addition to the culture medium has no effect on this activity in the *lasR* mutant, it induces significant and consistent levels of β-galactosidase activity in the wild-type strain (Fig. 14). Similar results are also observed with an *hcnA'-lacZ* fusion. These findings demonstrate that HHQ can induce the expression of genes that are also activated by PQS (Gallagher et al., *J Bacteriol* **184,** 6472-80, 2002), and this induction appears to require HHQ conversion into PQS, since no induction is found in cells that cannot carry out this conversion. Thus, HHQ does not itself function as a signal in our assay, independent of PQS activity, but instead is able to act as a messenger molecule that is converted into PQS by cells other than those that produce it.

Reduced pathenogenicity of *pqs* operon mutants

mvfR and *pqsB* mutants display reduced pathogenicity in plants, nematodes, insects, and mice (see references Wells, *J. Biol. Chem.* **196,** 331-340, 1952; Rahme et al., *Proc. Natl. Acad. Sci. USA* **94,** 13245-13250, 1997; 47, 48). When using the burn mouse model for mammalian pathogenicity assessment, PA14 induces a mortality rate of near 100 %, whereas its isogenic *mvfR* mutant gives only about 35 % mortality (Cao et al., *Proc Natl. Acad. Sci. U S A* **98,** 14613-8, 2001). Accordingly, we found here the *pqsA* and *pqsE* mutants also displayed attenuated mice virulence, with mortality rates of 40 % ± 3 and 38 % ± 12, respectively, thus confirming the importance of these *mvfR*-regulated genes in mammalian pathogenesis. This reduced pathogenicity presumably results from the down-regulation of QS-controlled virulence factors that require PQS synthesis (see references: McKnight et al., *J. Bacteriol.* **182,** 2702-2708, 2000; Lépine et al., *Biochim. Biophys. Acta* **1622,** 36-40, 2003), and/or a reduction of *pqsE* expression (Rahme et al., *Science* **268,** 1899-1902, 1995). The *pqsE* mutant, which produces wild-type HAQ levels, also exhibits attenuated virulence (Fig. 4 and Wells, *J. Biol. Chem.* **196,** 331-340, 1952); therefore PQS might not be directly relevant to pathogenicity.

pqsE* does not participate in extracellular HAQ synthesis, but is essential for the QS regulatory activity of *mvfR

Whereas *pqsA* or *pqsB* inactivation abolish HAQ production, a *pqsE* mutant produces essentially normal HAQ levels (Fig. 12), and HAQ-related antibiotic activity (Fig. 6). Indeed, a detailed kinetic analysis shows that *pqsE* inactivation has no effect on HAQ production (Fig. 4). In contrast, both its pyocyanin production and mouse pathogenicity are as attenuated as seen with the *mvfR* or other *pqs* mutants. Moreover, PQS addition to cultures of any *pqs* or *mvfR* mutants, or induction of PQS synthesis by HHQ addition, failed to induce pyocyanin production, potentially because of the lack of *pqsE* expression in these mutants. This was unexpected since the QS regulatory activity of *mvfR* was hypothesized to reflect the lack of PQS synthesis, suggesting that the presence or production of PQS is insufficient to activate pyocyanin synthesis, and perhaps other *mvfR*-controlled pathogenicity-related activities, whereas *pqsE* expression is essential. Supporting this notion, *mvfR* overexpression gave excessive pyocyanin accumulation (Fig. 12). BLAST analysis

and genomic context suggest that *pqsE* encodes an enzyme, perhaps a hydrolase, which converts PQS congeners into intracellularly active derivatives. Such compounds would not be detected by our assay, which was aimed at extracellular compounds. Loss of these compounds might underlie the reduced pathogenicity of a *pqsE* mutant.

Thus, using LC/MS and DNA microarray analyses, we have determined that the transcriptional regulator MvfR, originally identified via its requirement for full *P. aeruginosa* broad-host virulence, regulates the expression of *pqsABCDE* and *phnAB*, which encode enzymes involved in the synthesis of five distinct families of structurally related HAQ congeners. By adding labeled HAQ precursors to bacterial cultures, we have found that AA, mostly the product of the PhnAB synthase, is the precursor of all HAQs, and have therefore established the sequence of their synthesis. Significantly, we show that PQS synthesis requires an activity whose regulation depends on LasR, versus MvfR. Our results also revealed that one HAQ, HHQ, is the precursor of the PQS signaling molecule, and is itself both released from and taken up by bacteria, implicating it as an intercellular message molecule. These results provide insights into the structure, biosynthesis, regulation, and function of HAQs, and have allowed us to uncover a 'conversational' cell-to-cell communication pathway used by *P. aeruginosa*.

At least two branch pathways appear to have evolved from an ancestral synthetic HAQ pathway - one leading to the production of the antibacterial and cytochrome inhibitor *N*-oxide derivatives, and the second leading to PQS signaling congeners. Many HAQs have been previously identified as *P. aeruginosa* secondary metabolites (Budzikiewicz, *FEMS Microbiol. Rev.* **104,** 209-228, 1993; Leisinger et al., *Microbiol Rev* **43,** 422-42, 1979). We have confirmed that a number of these compounds have Gram-positive antimicrobial activity, and have attributed some of this activity to HQNO, the most abundant HAQ. Interestingly, this activity has been associated with the clearance of *S. aureus* lung colonization by *P. aeruginosa* (Machan et al., *J. Antimicrob Chemother* **30,** 615-623, 1992). HHQ is found in cystic fibrosis (CF) lung exudates (Machan et al., *J. Antimicrob. Chemother.* **30,** 615-623, 1992) and PQS occurs in the sputum and bronchoalveolar lavage fluid of CF lungs (Collier et al., *FEMS Microbiol. Lett.* **215,** 41-6, 2002), indicating that HAQs are produced *in vivo*. Guina *et al.* have reported that *P. aeruginosa* isolates from CF

patients produce more PQS than isolates from other diseases, suggesting that the PQS pathway could be upregulated in CF strains (Guina et al., *Proc. Natl. Acad. Sci. U S A* **100**, 2771-2776, 2003). Thus, *P. aeruginosa*, when colonizing novel infectious niches, may increase HAQ production to inhibit the growth of competing microorganisms.

Our study provides novel insights into PQS signaling, which functions in the expression of QS-regulated genes. Our results demonstrate that this system acts via at least two distinct extracellular molecules: PQS, and its precursor, HHQ. As illustrated in Fig. 6, this pathway can be viewed as 'conversational' cell-to-cell communication, since an HHQ molecule released by a cell in a population is taken up by another cell and converted into PQS, where it is then released into the extracellular milieu, to signal cells in the population. This signaling is not artifactual and that HHQ and PQS could mean different things to cells in a population, even if their ultimate signaling mechanisms are the same. For instance, their concentrations peak at different growth stages; their production is under different regulation (MvfR for HHQ, LasR for PQS); and they are likely produced in different cellular compartments. Indeed, the PSORT program, predicts that the PqsH monoxygenase, which probably mediates the hydroxylation of HHQ into PQS, is localized to the periplasmic space. This suggests that the HHQ that is converted to PQS typically has an extracellular versus intracellular origin, and engenders the question of whether the intracellular HHQ is destined to serve as the PQS precursor.

Cells in a bacterial community need to tell each other about their different properties, including their density, growth state, and production levels of extracellular compounds, such as antibiotics and virulence factors, in order to coordinate their activity. Presumably, different signals are required to convey this different information, and *P. aeruginosa* populations likely employ panoply of extracellular molecules. For instance, although HHQ is the precursor of PQS, these two molecules could convey different information: HHQ reflects the extracellular levels of HAQ including antimicrobial and cytochrome inhibitory functions, and indicates the level of MvfR activity; while PQS reflects LasR activity levels and the status of the AHL-based QS system in population growth regulation. Also, that HAQ and PQS levels peak at different times suggests that PQS-mediated signaling reflects HAQ levels at one growth stage, and PQS levels at another.

Finally, HHQ and PQS analogues are largely cell-associated (Lépine et al., *Biochim. Biophys. Acta* **1622**, 36-40, 2003), suggesting the possibility that HAQ-based intercellular communication is mediated through cell-to-cell contact. Such contact and intercellular communication may be particularly important in chronic *P. aeruginosa* infections, including those of the lungs of CF patients, as it is beneficial for bacteria to regulate their populations in such niches. Significantly, both HHQ and PQS are produced in the lungs of CF individuals (see references: Machan et al., *J. Antimicrob. Chemother.* **30**, 615-623, 1992; Collier et al., *FEMS Microbiol. Lett.* **215**, 41-6, 2002), suggesting that these molecules represent a pharmacological target for treating the debilitating *P. aeruginosa* infections common to CF patients.

PQS is required for the *activation* of *pqsA-E* operon

To demonstrate that PQS indeed is an inducer, *pqsA* mutant cultures carrying the *pqsA-lacZ* reporter were exposed to PQS, anthranilic acid (AA), and complete supernatant extract (used in Fig. 8), and LacZ activity was measured. Fig. 15 demonstrates that PQS activates *pqsA* transcription in the mutant background (*pqsA*$^-$), in the absence of HAQ production. As demonstrated, the ethyl acetate extract (EA extract) containing all HAQs and PQS congeners, also induces expression, while AA, the PhnAB product and precursor of all HAQs does not.

***lysR-box* is critical for *pqsA-E* transcriptional activation**

Our transcriptome studies show MvfR is essential for the transcription of the *pqsA-E* operon. This operon carries a putative *lysR* box centered at -45 bp, relative to the *pqsA* transcription initiation site; and two putative *las*-box specific palindromic sequence, centered at -311 bp and -151 bp, respectively (Fig. 16A), suggesting that the LasR and/or the RhlR proteins, in addition to MvfR, may bind to the *pqsA* promoter to regulate *pqsA-E* transcription. To determine whether the three putative control elements function in *pqsA-E* regulation, we generated deletion mutants in the *pqsA-E* regulatory region according to srandard methods (Fig. 16). The control plasmid pGX5 contains the segment from -486 relative to the *pqsA* transcription initiation site through the first 160 (+231) nucleotides of the *pqsA* gene, and thus presumably the complete *pqsA-E* regulatory region, fused to *lacZ*. Beginning with this construct, we then deleted the regions from -486 to -247 bp, -486 to -90 bp, and -486

to -33 bp, to respectively generate pGX6, pGX7, and pGX8. These constructs were then separately introduced into PA14 to assess the importance of the putative *las*-boxes and *lysR*-box. Fig. 16B shows that the β-gal activity of pGX5 increases with PA14 cell density; and that deletion of the -486 to -318 bp putative *las*-box results in increased *pqsA* transcription, suggesting that a transcription factor(s) binds here to repress *pqsA* transcription. To determine if the *las*-box functions in *pqsA* repression, we used PCR site-directed mutagenesis (Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1997. Short protocols in molecular biology, 3rd ed. John Wiley & Sons, Inc., New York, N.Y.) on pGX5 to replace one of the most conserved *las*-box nucleotides (Whiteley et al., *J. Bacteriol.* **183**,5529-5534, 2001), position 3C, with T (highlighted in grey, Fig. 16A). This substitution does not significantly alter *pqsA* activation (Fig. 16B). Similarly, deletion of the -317 to -90 bp region does not alter *pqsA* expression. These results indicate that neither of the putative *las*-boxes acts in *pqsA* regulation. In contrast, no *pqsA* expression occurs when the putative *lysR*-box is deleted, indicating that the region between -89 and -33 bp is essential for *pqsA* activation.

To further examine the role of the *lysR*-box, we modified pGX7 by replacing one of the most conserved nucleotides of the *lysR*-box, position 1 T, with an A, to generate pGX9; and deleted half of the putative symmetric *lysR* box, to generate pGX10 (Fig. 16A; *T1A* and *half*, respectively). These plasmids were then transformed into PA14. Fig. 16C shows that the *lysR*-box is an essential regulatory element for *pqsA* activation: the T to A substitution severely reduces, while the half *lysR*-box deletion completely abrogates, *pqsA* expression, respectively. This also suggests that the LysR-type transcription factor MvfR activates *pqsA-E* transcription by binding to the *lysR*-box, in agreement with our transcriptome data (13), and the observation that *pqsA* expression is abolished in the *mvfR* mutant (Fig. 16D).

The above experiments were performed using the following materials and methods.

Materials and Methods

Bacterial strains, plasmids and media.

P. aeruginosa strains include: wild type PA14 (Rahme et al., *Science* **268,** 1899-1902, 1995); an *mvfR* mutant (Cao et al., *Proc. Natl. Acad. Sci. USA* **98,** 14613-8, 2001); 8C12, a Tn*phoA*-insertion mutant of *pqsB* (Mahajan-Miklos et al., *Cell* **96,** 47-56, 1999); and a *lasR*::Gm mutant, which was generated by allelic exchange using pSB219.9A as described (Beatson et al., *J Bacteriol* **184,** 3598-604, 2002). A *pqsE* deletion mutant was generated via pEX18Ap allelic replacement, using sucrose selection, resulting in a 570 bp non-polar deletion covering 65% of the sequence (Hoang et al., *Gene* **212,** 77-86, 1998). The *pqsA* (U479) Tn*phoA* mutant was obtained from the PA14 Transposon Insertion Mutant Database. For complementation analysis, *mvfR* was cloned into pDN18 (Nunn et al., *J. Bacteriol.* **172,** 1990). The reporter fusions *phzABC-lacZ* and *hcnA'-lacZ* have been described (Whiteley et al., *J. Bacteriol.* **182,** 4356-60, 2000; Pessi et al., *J. Bacteriol.* **182,** 6940-9, 2000). Plasmids were transformed into PA14 by electroporation (Smith et al., *Nuc. Acid. Res.* **17,** 10509, 1989). Specific β-galactosidase activity was determined as reported (Miller, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 352-355 1972).

Bacteria were grown in Luria-Bertani broth or on 1.5 % Bacto-agar (Difco) LB plates. Freshly plated cells served as inoculum. For pyocyanin production, bacteria were grown in King's A broth (King et al., *J. Lab. Clin. Med.* **44,** 301, 1954) and the pyocyanin quantified as OD_{520} after supernatant extraction (Essar et al., *J. Bacteriol.* **172,** 884-900, 1990). Tetracycline (75 mg/L), carbenicillin (300 mg/L), kanamycin (200 mg/L), and gentamicin (100 mg/L) were included as required.

LC/MS analysis

Analyses were performed using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass Canada, Pointe-Claire, Can.) in positive electrospray ionization mode, interfaced to an HP1100 HPLC equipped with a 4.5 x 150 mm reverse phase C_8 column. Culture supernatants were twice extracted with ethyl acetate, the solvent was evaporated, and the residue was dissolved in a

water/acetonitrile mixture containing the internal standard. Alternatively, culture samples were directly diluted with a methanolic solution of the internal standard, as reported (Lépine et al., *Biochim. Biophys. Acta* **1622,** 36-40, 2003).

Synthesis of labeled HAQ

4-hydroxy-2-heptylquinoline *N*-oxide (HQNO) was from Sigma. 2,3,4,5-tetradeuteroanthranilic acid (AA-d_4) was from CDN isotopes (Pointe-Claire, Canada). The internal standards, 5,6,7,8-tetradeutero-3,4-dihydroxy-2-heptylquinoline (PQS-d_4), and 5,6,7,8-tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d_4) were synthesized as reported (Lépine et al., *Biochim. Biophys. Acta* **1622,** 36-40, 2003). 5,6,7,8-tetradeutero-4-hydroxy-2-heptylquinoline *N*-oxide (HQNO-d_4) was synthesized from HHQ-d_4 (Cornforth et al., *Biochem. J.* **63,** 124-130, 1956).

RNA isolation and transcriptome analysis

Whole genome expression profiles were produced in duplicate for PA14 and the *mvfR* mutant. Cultures were grown in 1 L Erlenmeyer flasks with 100 ml LB at 37°C and 200 rpm. Cells were sampled at OD_{600} = 1.5, 2.5, 3.5 and 4.5, and their RNA was immediately stabilized with RNAprotect Bacteria Reagent (Qiagen, Valencia, CA) and stored at –80°C. Total RNA was isolated with the RNeasy spin column (including an on-column DNase digestion step) according to the manufacturer (Qiagen), treated with RQ1 DNAse I (Promega, Madison, Wis.) for 1 hr at 37°C, and repurified through an RNeasy column.

Samples were labeled according to the manufacturer (Affymetrix), and hybridized to the Affymetrix GeneChip® *P. aeruginosa* Genome array for 24 hrs at 50°C using the GeneChip® hybridization oven at 60 rpm. Washing, staining, and scanning were performed according to Affymetrix. The original data files, obtained from the array scans hybridized with the different probes, were converted to cell intensity files (.CEL files) using the Microarray Suite 5.0. Data analysis/clustering was performed with the DNA-Chip Analyzer (dCHIP) software (Li et al., *Proc. Natl. Acad. Sci. USA* **98**, 31-36, 2001).

Antimicrobial activity assay

HAQ antimicrobial activity was evaluated on well-plates. An overnight culture (30 µl) was plated to produce a bacterial lawn, and 5 mm diameter holes were punched in the agar and filled with 60 µl of a 25% methanol solution of test extract or pure HAQ. Plates were incubated overnight at 37°C and scored for growth inhibition zones around the test wells.

Cell-to-cell communication assay

To test if *P. aeruginosa* cells produce PQS in response to HHQ released by other cells, we compared the concentrations of PQS in cultures, grown in 30 ml LB in 250 ml flasks, of a *lasR* mutant, to co-cultures containing 50% of a *lasR* mutant and an *mvfR* mutant. pDN18*mvfR* was introduced into the *lasR* mutant to compensate for the lower expression of *mvfR* in this background. The effect on gene expression of exogenous HHQ was assayed by comparing the β-galactosidase activity of PA14 versus *lasR*⁻ cells carrying the *phzABC-lacZ* or *hcnA'-lacZ* fusions, grown in the absence or presence of 10 mg/L of HHQ.

Other Embodiments

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is: